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Canonical WNT/β-catenin signaling is required for ureteric branching

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Abstract

WNT/β-catenin signaling has an established role in nephron formation during kidney development. Yet, the role of β-catenin during ureteric morphogenesis in vivo is undefined. We generated a murine genetic model of β-catenin deficiency targeted to the ureteric bud cell lineage. Newborn mutant mice demonstrated bilateral renal aplasia or renal dysplasia. Analysis of the embryologic events leading to this phenotype revealed that abnormal ureteric branching at E12.5 precedes histologic abnormalities at E13.5. Microarray analysis of E12.5 kidney tissue identified decreased *Emx2* and *Lim1* expression among a small subset of renal patterning genes disrupted at the stage of abnormal branching. These alterations are followed by decreased expression of genes downstream of *Emx2*, including *Lim1*, *Pax2*, and the ureteric tip markers, *c-ret* and *Wnt 11*. Together, these data demonstrate that β-catenin performs essential functions during renal branching morphogenesis via control of a hierarchy of genes that control ureteric branching.

Keywords: β-Catenin; Kidney development; Branching morphogenesis; WNT signaling; Canonical signaling; Microarray; *Emx2*

Introduction

Branching morphogenesis, defined as growth and branching of epithelial tubules during embryogenesis, is critical to the formation of organs including the kidney, lung, and mammary glands. In each organ, a multi-branched epithelial tree develops from unbranched epithelial progenitors in response to signals elaborated from adjacent mesenchymal tissue. Formation of the mammalian permanent kidney (metanephros) begins with the emergence of the ureteric bud as an epithelial outgrowth of the Wolffian duct in response to the adjacent metanephric mesenchyme. The ureteric bud invades the metanephric mesenchyme and gives rise to successive generations of ureteric bud branches that differentiate into the collecting ducts. In reciprocal fashion, metanephric mesenchyme cells adjacent to ureteric bud tips undergo a series of morphologic transitions whereby the epithelial elements of the nephron are formed (Saxen, 1987).

The stereotypic pattern of collecting duct and nephron formation suggests that the embryologic events giving rise to these structures are tightly regulated. Yet, the signaling mechanisms that control branching morphogenesis during mammalian kidney development (reviewed in Vainio and Lin, 2002) are not completely defined. β-Catenin is a bifunctional protein involved in cell–cell adhesion via adherens junctions, and in gene transcription as a critical effector in the canonical WNT pathway. Members of the WNT family of secreted glycoproteins control tissue patterning during vertebrate embryogenesis (Nusse, 2005). WNT signaling regulates cell functions by activating the canonical β-catenin pathway, the planar cell polarity pathway or the calcium pathway. WNT signals control levels of cytosolic β-catenin by inhibiting Glycogen Synthase Kinase (GSK) 3β-dependent β-catenin phosphorylation and subsequent proteasomal degradation of β-catenin. Inhibition of GSK3β

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allows accumulation of β-catenin, which causes its nuclear translocation. In the nucleus, β-catenin regulates target gene expression in partnership with members of the Leukemia and the T Cell Factor (LEF/TCF) family (Huelsken and Behrens, 2002). The expression of *Wnt6, Wnt7b, Wnt9b and Wnt11* in the ureteric bud is consistent with a functional role for these WNTs during ureteric branching. Expression of β-catenin-dependent reporter genes in the Wolffian Duct and ureteric bud in transgenic mice suggests that WNTs can signal via the canonical pathway during branching morphogenesis (Maretto et al., 2003; Moriyama et al., 2007). While analysis of mice with β-catenin deficiency or over expression in nephron progenitors demonstrates an important role β-catenin during nephrogenesis (Park et al., 2007), the role of β-catenin and canonical Wnt signaling during ureteric branching is not defined.

Here, we demonstrate an essential role for β-catenin during renal branching morphogenesis. *Cre*-mediated inactivation of β-catenin targeted to ureteric cells disrupts branching morphogenesis and causes renal aplasia or dysplasia. Genome-wide analysis of mRNA expression and in situ mRNA hybridization in mutant and wild type kidney tissue demonstrated that decreased ureteric branching observed during the early stage of renal malformation in β-catenin mutant mice is accompanied by decreased *Emx2* and *Lim1* expression. These changes are followed by decreased expression of *Pax-2* and *c-ret* and the c-ret-dependent gene, *Wnt11*. Together, these data demonstrate that β-catenin acts upstream of a hierarchy of genes required for ureteric branching.

Materials and methods

Mice

Mice with a β-catenin allele containing LoxP sites flanking exons 2 through 6 (Brault et al., 2001) were crossed to *Hoxb7-Cre:Gfp* mice (Zhao et al., 2004) to generate *Hoxb7-Cre,Gfp*; *β-catenin* deficient mice. PCR genotyping was performed as described (Brault et al., 2001). TCF- and BAT-gal reporter mice (Cheon et al., 2002; Maretto et al., 2003) were used to determine β-catenin transcriptional activation. β-Galatosidase staining was performed as previously described (Godin et al., 1998).

Histology and immunohistochemistry

Paraffin-embedded embryos were analyzed by histology after generating 4 μm tissue sections and staining with haematoxylin and eosin. Anti-β-catenin C-18 (1:200 dilution, Santa Cruz, USA), and E-cadherin (1:100 dilution, Zymed, USA) immunohistochemistry was performed on formalin-fixed, paraffin-embedded kidney sections as previously described (Hu et al., 2003). Immunofluoresence was performed using anti-β-catenin (1:100 dilution) (Upstate, Lake Placid, USA) and anti-calbindin (1:200 dilution) (Sigma). Whole mount immunofluoresence was performed as described (Kuure et al., 2005) with DBA lectin (Vector Laboratories; 1:200) to identify the stalk epithelium (Michael et al., 2007). Alexa 568 and Alexa 488 (1:500 dilution) were used as secondary antibodies (Invitrogen, St. Louis, USA).

Microarray analysis

Kidneys from mutant mice were divided into three random pools ($n=3$) consisting of 6 kidneys each, while those from wild type mice were divided into 3 pools ($n=3$) consisting of 3 kidneys each. Kidneys were stored in RNAlater RNA stabilization reagent (Qiagen) and RNA was then isolated using the RNeasy micro kit (Qiagen). This provided sufficient RNA (1 μg total RNA) for a one cycle amplification. Microarray data was processed using GCOS (v1.4, Affymetrix). All CHIPS were scaled to a target value of 500 prior to expression analysis. Two wild type replicates were normalized to a third wild type replicate and these were used as baselines to normalize and compare the three replicate β-catenin deficient samples. Comparisons were made in all combinations to create a matrix of 3×3 crosswise comparisons (9 in total). Probe sets with present calls in all replicates in either the β-catenin deficient or wild type samples were kept and all others were removed from the data set. Probe sets with a significant change call in 6 of 9 comparisons were considered significantly changed in the β-catenin deficient versus wild type samples. To minimize the false positives a threshold signal log ratio (SLR) value was determined by estimating the background error using the distribution of SLR for probe sets with no significant change calls.

Gene ontology analysis

Probe sets were mapped to their gene symbol identifiers from annotation tables supplied by Affymetrix (http://www.affymetrix.com). Lists of gene symbols were entered into the BINGO plugin [v2.0 (Maere et al., 2005)] for Cytoscape [v 2.4 (Shannon et al., 2003)] and compared to Gene Ontology (GO) annotation tables from MGI (http://www.informatics.jax.org/) to calculate the enrichment of terms and their *P*-value, which was then adjusted by the Benjamini Hochberg correction for the false discovery rate (Hochberg and Benjamini, 1990). All reported enrichments were at a significance of 0.05 or less.

Real-time reverse transcriptase-PCR

Real-time PCR Amplification was performed using the Applied Biosystems 7900HT fast RT-PCR system. cDNA was generated using first strand cDNA synthesis (Invitrogen) from total RNA. Real-time PCR reaction mix contained 3 ng of each cDNA sample, SYBR green PCR Master Mix (Applied Biosystems) and 300 nM of each primer to a total volume of 25 μl. Primers for *Emx2*, *Lim1*, *c-ret*, *Wnt11*, *Pax2*, *myogenin*, and *E-cadherin* were designed using Primer 3 software and verified using the UCSC genome bioinformatics web site (genome.ucsc.edu). Primer design was very restrictive — the annealing temperature was restricted to 59–60 °C and the length of the PCR product was set between 100 and 200 bp. Specificity of the amplification was carried out by agarose gel electrophoresis. Relative levels of mRNA expression were carried out using the standard curve method. Individual expression values were normalized by comparison to β-2-microglobin.

Electron microscopy

Kidneys were fixed in 2% glutaraldehyde (GA) for 24 h, rinsed with 0.1 M sodium cocadylate and fixed for 1 h in 0.2% tannic acid followed by graded fixation in 1% osmium tetroxide and in 1% osmium tetroxide/1.25% potassium ferrocyanide. After dehydration samples were embedded in SPURR resin, sectioned, collected on copper grids and stained for EM. Images were obtained using a FEI Tecnai 20 transmission electron microscope.

In situ mRNA hybridization

Non-radioactive in situ hybridization was performed using DIG-labeled cRNA probes encoding *Emx2*, *Lim1*, *Pax-2*, *c-ret*, *GDNF, Wnt 11* on paraffin-embedded kidney tissue fixed with 4% PFA for 24 h at 4 °C as previously described (Mendelsohn et al., 1999).

In situ TUNEL assay, BrdU incorporation and data analysis

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using 4% PFA fixed paraffin-embedded tissue sections as described in the manufacturer's instructions (Promega, Madison, WI). Cell proliferation was assayed in paraffin-embedded kidney tissue by incorporation of 5-bromo-2-deoxyuridine (BrdU, Roche Molecular Biochemicals, Mannheim, Germany), as described (Cano-Gauci et al., 1999). Pregnant